

Specific Binding of Tryptophan Transfer RNA to Avian Myeloblastosis Virus RNA-Dependent DNA Polymerase (Reverse Transcriptase)

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Notes:

Specific binding of tryptophan transfer RNA to avian myeloblastosis virus RNA-dependent DNA polymerase (reverse transcriptase)

(primer/DNA synthesis/70S RNA/RNA tumor virus)

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ABSTRACT The ability of tryptophan tRNA (tRNA^{Trp}) to initiate reverse transcription of the 70S RNA of avian RNA tumor viruses suggested that the reverse transcriptase (RNA-dependent DNA polymerase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase; EC 2.7.7.7) might have a specific binding site for the tRNA. A complex of tRNA^{Trp} and the avian myeloblastosis virus reverse transcriptase has been demonstrated using chromatography on Sephadex G-100 columns. Of all the chicken tRNA's, only tRNA^{Trp} and a tRNA^{Met} bind to the enzyme with high enough affinity to be selected from a mixture of the chicken cell tRNAs. The ability of tRNA^{Trp} to change the sedimentation rate of the enzyme indicates that tRNA^{Trp} is not binding to a contaminant in the enzyme preparation. Treatment of the enzyme with monospecific antibody to reverse transcriptase prevented binding of tRNA as well as inhibited the DNA polymerase activity of the enzyme. The ability of reverse transcriptase to utilize tRNA^{Trp} as a primer for DNA synthesis, therefore, appears to involve a highly specific site on the enzyme.

In vitro synthesis of DNA by the DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) found in virions of RNA tumor viruses (reverse transcriptase) is a primer-dependent reaction (1, 2). When the "70S" RNA from virions is used as a template, a low-molecular-weight RNA of the 70S complex acts as primer (3-5). For Rous sarcoma virus, this RNA has been shown to be identical to a cellular tryptophan tRNA (tRNA^{Trp}) that is called "spot 1 RNA" after separation by 2-dimensional gel electrophoresis (6-9). Such tRNA^{Trp} has been isolated from three sources: 70S RNA, free 4S RNA from virions of Rous sarcoma virus, and normal chicken embryo fibroblasts (4, 5, 8).

Although other tRNAs are found associated with 70S RNA and as free 4S RNA in virions (10), 90% of the DNA molecules copied from 70S RNA have been shown to be initiated using tRNA^{Trp} as a primer (4). Part of its ability to act as primer may result from the tight association of tRNA^{Trp} with the 35S RNA subunits of the 70S RNA; of the 70S-associated tRNAs, tRNA^{Trp} requires the highest temperature for removal from the 35S RNA (4, 11). The tRNA^{Trp} also appears to be able to anneal back to 35S RNA (12, 13). The association of tRNA^{Trp} with 35S RNA, however, is not sufficient to explain its ability to act as primer for the reverse transcriptase because, in general, the enzyme uses DNA efficiently as primer but utilizes RNA primers poorly (1, 14). The apparent selectivity of reverse transcriptase for tRNA^{Trp} over other 70S-associated tRNAs and the poor rate of priming of reverse transcriptase by RNA homopolymers led us to investigate whether there might be a specific binding site on the reverse transcriptase for tRNA^{Trp} .

Abbreviation: AMV, avian myeloblastosis virus.

Using an assay for high-affinity binding sites, we have been able to demonstrate specific binding of tRNA^{Trp} to the reverse transcriptase of avian myeloblastosis virus (AMV).

MATERIALS AND METHODS

Materials. AMV reverse transcriptase was purified by sequential chromatography on columns of DEAE-Sephadex and phosphocellulose (15). The phosphocellulose fraction was further purified on a DNA-cellulose column (16). The enzyme preparation gave only two bands after electrophoretic separation on 10% sodium dodecyl sulfate-polyacrylamide gels; these migrated as do the previously characterized α and β subunits of AMV reverse transcriptase (17). Antiserum to AMV reverse transcriptase was obtained from Dr. R. Nowinski, Madison, Wisc. (18). The IgG fraction was purified from serum as described (19). Chicken liver tRNA and chicken liver aminoacyl tRNA synthetases were prepared following published procedures (20-22). Purified *Escherichia coli* tRNA^{Tyr} , *E. coli* tRNA^{Ser} , and yeast tRNA^{Phe} were gifts from Dr. U. L. RajBhandary and M. Silberklonig. $(\text{dG})_{12-18}$ and $(\text{dT})_{12-18}$ were purchases from Collaborative Research Corp. These polynucleotides were labeled at their 5'-end using polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (23). Radioactive nucleotides and amino acids were products of New England Nuclear Corp. Sephadex G-100 was obtained from Pharmacia Corp.

Preparation of ^{32}P -Labeled RNAs. Cultures of chicken embryo fibroblasts infected with Schmidt-Ruppin D Rous sarcoma virus or Carr-Zilber associated virus were obtained from Dr. H. M. Temin. The cells were radioactively labeled in 100 mm diameter dishes with $[\text{}^{32}\text{P}]\text{orthophosphate}$ as described previously (7), using 1-2 mCi of $[\text{}^{32}\text{P}]\text{orthophosphate}$ per ml of medium. After 48 hr of labeling, virus was harvested from the medium by centrifugation and RNA was extracted without further purification (7). Viral RNA was fractionated into 70S and "free" 4S RNA by centrifugation through sucrose gradients (7). The 4S RNA was precipitated with 2 volumes of ethanol, resuspended in 0.05 M NaCl, 0.01 M Tris-HCl, pH 7.8, 0.01 M MgCl_2 , and was applied to a 1 ml column of DEAE-Sephadex A-50 (Pharmacia). After successive washes with 0.05 M NaCl and 0.3 M NaCl the RNA was eluted from the column with 1 M NaCl in the same buffer and concentrated by ethanol precipitation.

After virus was harvested, the labeled cells were washed twice with 5 ml of TSE buffer (0.02 M Tris-HCl, pH 7.8; 0.1 M NaCl; 1 mM EDTA) at room temperature. Cells were then treated on the culture plates with 0.5% sodium dodecyl sulfate in TSE buffer, 1 ml/10 cm plate for 15 min at room temperature. The suspension was carefully removed from

the plates to avoid shearing of DNA and 0.25 volume of 5 M NaCl was added. After storage overnight, the precipitate was removed by centrifugation at $15,000 \times g$ for 20 min. The supernatant was extracted twice with phenol and twice with chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated with 2 volumes of ethanol at -20° in the presence of 100 μ g of carrier RNA (phenol-extracted Sigma type IV yeast RNA). RNA was purified by chromatography on DEAE-Sephadex as described above, followed by centrifugation in a 5–20% sucrose gradient in TSE buffer containing 0.5% Sarkosyl (Geigy) at 49,000 rpm, 2 hr, 4° in a Beckman SW 50.1 rotor.

The individual tRNA species were purified from virion-associated or cellular tRNA using 2-dimensional gel electrophoresis as described previously (7).

Unlabeled tRNA^{Trp} from chicken liver was purified as described elsewhere (9). *E. coli* 32 P-labeled 5S RNA and 4.5S RNA were prepared as described previously (6) using the hot sodium dodecyl sulfate method of Bremer and Yuan (24).

Binding Assay. For assay of binding, the reaction mixture (100–200 μ l) contained 50 mM Tris-HCl (pH 8.3), 60 mM NaCl, 10 mM 2-mercaptoethanol, 10 mM MgCl₂, 5% (v/v) glycerol, 0.1% Nonidet P-40, [32 P]RNA as indicated, and 50 to 150 enzyme units of reverse transcriptase. After 10 min incubation at 2° , the reaction mixtures were chromatographed on columns of Sephadex G-100 (0.6 \times 23 cm) pre-equilibrated at 4° with buffer A (0.1 M potassium phosphate, pH 7.5, 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol, 0.1 mM EDTA, and 0.1% Nonidet P-40). The column was eluted with buffer A. Fractions of 0.17 ml were collected and 25–50 μ l portions of each fraction were assayed for reverse transcriptase activity; 60–70% of added enzymatic activity was routinely recovered in the fractions. Portions of each of the fractions were also used for determination of [32 P]RNA.

Enzyme Assay. Reaction mixtures (100 μ l) contained 50 mM Tris-HCl (pH 8.3), 6 mM MgCl₂, 0.1 mM [3 H]dGTP (100 cpm/pmol), 2 μ g of poly(C), 0.5 μ g of (dG)_{12–18}, 5 mM dithiothreitol, and 25–50 μ l of fractions from the Sephadex G-100 columns or glycerol gradients. Reactions were incubated for 30 min at 37° . One enzyme unit was defined as the amount needed to catalyze the incorporation of 1 pmol of dGMP into polymer in 1 min. The assay for *E. coli* DNA polymerase I was as described for reverse transcriptase but 1 μ g of poly(dA) and 1 μ g of (dT)_{12–18} were used as the template and primer and the incorporation from [3 H]dTTP (60 cpm/pmol) was followed.

RESULTS

Binding of tRNA^{Trp} to Reverse Transcriptase. To study its binding to the AMV reverse transcriptase, we chromatographed [32 P]tRNA^{Trp} purified from chicken cells or from virions of an avian leukosis virus through Sephadex G-100 with or without added reverse transcriptase. When chromatographed by itself, tRNA^{Trp} eluted from the column as an included peak of radioactivity (Fig. 1A). When mixed with reverse transcriptase, most of the [32 P]tRNA^{Trp} eluted in the excluded volume and no peak of included tRNA^{Trp} was evident (Fig. 1B). The reverse transcriptase, a protein of 160,000 daltons (17), was found in the excluded volume whether or not tRNA^{Trp} was added. About 30% of the 32 P in a preparation of [32 P]tRNA^{Trp} from chicken cells also eluted in the excluded volume from Sephadex G-100 when mixed with reverse transcriptase (Fig. 1C) but it was entirely in-

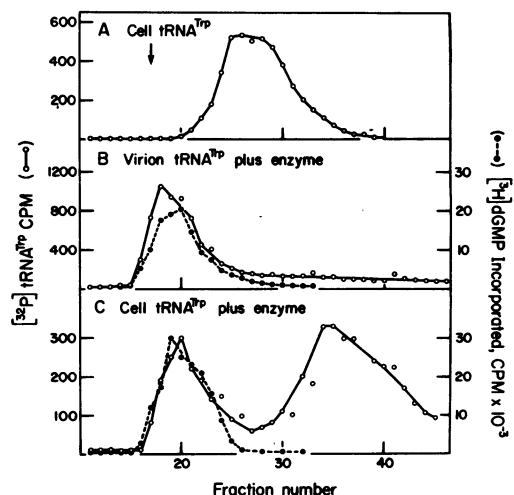


Fig. 1. Effect of reverse transcriptase on gel filtration pattern of [32 P]tRNA^{Trp}. Binding reactions and chromatography on Sephadex G-100 columns were carried out as described in *Materials and Methods*. (A) Cell [32 P]tRNA^{Trp} (3600 cpm), arrow marks the excluded volume of the column; (B) virion [32 P]tRNA^{Trp} (7300 cpm) and reverse transcriptase (50 units), and (C) cell [32 P]tRNA^{Trp} (4100 cpm) and reverse transcriptase (50 units). O—O, [32 P]tRNA; ●—●, reverse transcriptase activity.

cluded in the column if the enzyme was not added (not shown). This gel filtration assay is, therefore, capable of detecting a complex between the reverse transcriptase and tRNA^{Trp}. Attempts at using a Millipore filter binding assay have been unsuccessful.

Four different preparations of tRNA^{Trp} from chicken cells were studied for their binding to reverse transcriptase; about

Table 1. Binding of different RNA species to reverse transcriptase

32 P-labeled polynucleotide	Unbound* cpm	Bound*	
		cpm	% of total
Cell tRNA ^{Trp}			
without enzyme	3,600	0	0
Cell tRNA ^{Trp} †	3,190	2,330	42
Cell tRNA ^{Trp} ‡	2,040	1,030	34
Cell tRNA ^{Trp} †	3,150	1,440	32
Cell tRNA ^{Trp} ‡	4,490	1,810	29
Spot 2 virion tRNA	7,940	330	4
Spots 4 and 5			
virion tRNA	9,700	460	4
Chicken 5S RNA	9,620	500	5
<i>E. coli</i> tRNA ^{Tyr}	7,010	0	0
<i>E. coli</i> tRNA ^{Ser}	7,470	620	7
Yeast tRNA ^{Phe}	9,170	560	5
<i>E. coli</i> 4.5S RNA	5,380	570	9
<i>E. coli</i> 5S RNA	2,070	80	4
(dG) _{12–18}	74,490	490	0.6
(dT) _{12–18}	64,230	690	1

* Radioactivity in the excluded volume of a Sephadex G-100 column was considered "bound" to reverse transcriptase; radioactivity in the included volume was considered "unbound". Fifty units of reverse transcriptase were used in these assays.

† 32 P spot 1 from three different preparations of chick embryo fibroblasts.

‡ Twenty-fold less enzyme (2.5 units) was added in this reaction.

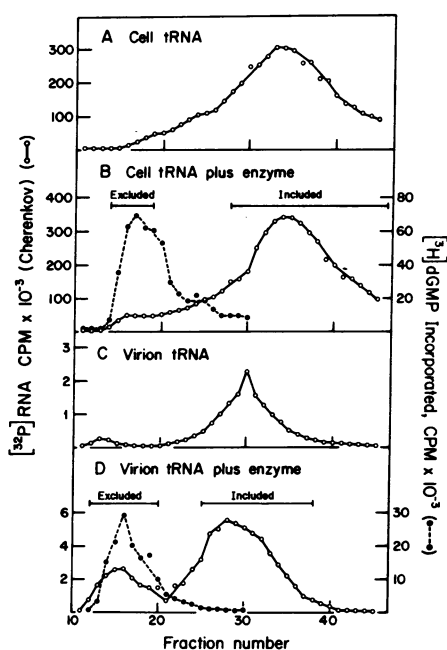


FIG. 2. Effect of reverse transcriptase on gel filtration pattern of unfractionated tRNA. (A) Cell $[^{32}\text{P}]$ tRNA (4.2×10^6 cpm). (B) Cell $[^{32}\text{P}]$ tRNA (10.6×10^6 cpm) and reverse transcriptase (150 units); (C) $[^{32}\text{P}]$ tRNA from virions of Schmidt-Ruppin D Rous sarcoma virus (14,600 cpm); (D) $[^{32}\text{P}]$ tRNA from Schmidt-Ruppin D Rous sarcoma virus (63,000 cpm) and reverse transcriptase (150 units). The excluded and included volumes from the columns were pooled separately for analysis by polyacrylamide gel electrophoresis (see Fig. 3). Symbols as in Fig. 1.

35% of each tRNA^{Trp} preparation bound to the enzyme as measured by the gel filtration assay (Table 1). Using 20-fold less enzyme than in standard assays reduced binding only slightly (Table 1) so the reactions appeared to be in enzyme excess. To determine whether the fraction of tRNA^{Trp} that would not bind to the enzyme was different from the bound fraction, ribonuclease T₁ digests of the $[^{32}\text{P}]$ RNA in the included and excluded volumes of the Sephadex G-100 column in Fig. 1C were fractionated by the methods of Sanger *et al.* (25) and their fingerprints were compared. The RNA preparations gave identical fingerprints and both fractions appeared to be greater than 90% pure tRNA^{Trp} (data not shown).

Specificity of Binding. A variety of purified RNAs have been investigated for their ability to bind to AMV reverse transcriptase. Three other tRNAs from virions of Rous sarcoma virus were tested and none showed significant binding (Table 1). Various *E. coli* or yeast tRNAs and *E. coli* or avian 5S RNAs did not bind detectably. Neither oligo(dT) nor oligo(dG) bound significantly to the enzyme even though they act as primers for the appropriate RNA templates (1).

Selection of tRNA^{Trp} by Reverse Transcriptase. The apparent specificity of reverse transcriptase for tRNA^{Trp} suggested that it might select only tRNA^{Trp} from a mixture of virion-associated or chicken cell tRNAs. Therefore, unfractionated $[^{32}\text{P}]$ tRNA from chicken embryo cell cultures or from Schmidt-Ruppin Rous sarcoma virus was mixed with reverse transcriptase at a ratio calculated to produce an approximate molar equivalence of enzyme protein to tRNA^{Trp}. All but about 2% of the cell tRNA preparation was included by the Sephadex G-100 column (Fig. 2A) and mixing reverse transcriptase with the tRNA increased the RNA in the excluded volume only slightly (Fig. 2B). Increasing the ratio of

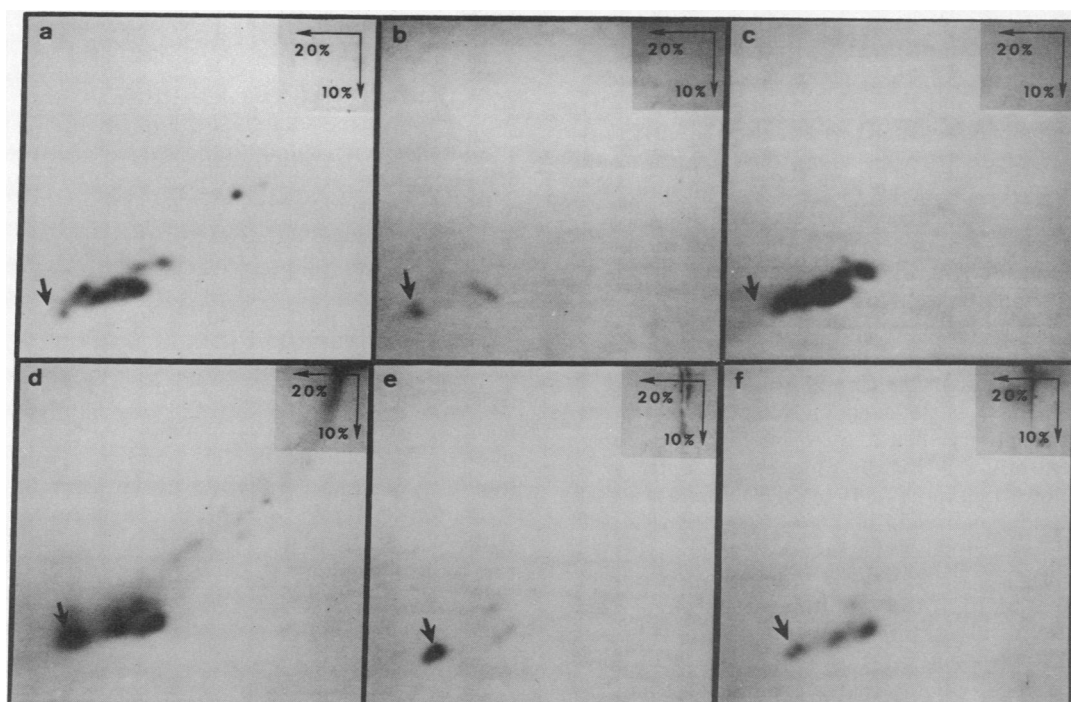


FIG. 3. Two-dimensional electrophoresis of cell or virion $[^{32}\text{P}]$ tRNA after gel filtration in the presence of reverse transcriptase, as described in ref. 7. (a) Cell $[^{32}\text{P}]$ tRNA before binding reaction; (b) cell tRNA bound by reverse transcriptase (excluded volume; Fig. 2B); (c) cell tRNA not bound by reverse transcriptase (included volume; Fig. 2B); (d) virion-associated $[^{32}\text{P}]$ tRNA before binding reaction; (e) virion tRNA bound by reverse transcriptase (excluded volume; Fig. 2D); (f) virion tRNA not bound by reverse transcriptase (included volume; Fig. 2D).

enzyme to tRNA did not increase the amount of bound tRNA (data not shown). Thus, a small proportion of total chick tRNA was bound to the enzyme. Of the virion-associated tRNA, little was excluded from the Sephadex G-100 in the absence of enzyme (Fig. 2C) but 33% bound to the enzyme (Fig. 2D).

To examine whether any specific tRNA might have been selected by the enzyme from chick tRNA, we analyzed the excluded and included fractions from Fig. 2A and 2B by 2-dimensional polyacrylamide gel electrophoresis (Fig. 3). Total chick tRNA contains a large number of species of tRNA separable by this method (Fig. 3a). The fraction of tRNA appearing in the excluded volume of a Sephadex G-100 column in the absence of added reverse transcriptase was not enriched for any of these tRNA species (data not shown). The fraction excluded from Sephadex in the presence of reverse transcriptase was greatly enriched for three tRNA species (Fig. 3b). The major enriched species (arrow in Fig. 3b) had the same mobility as tRNA^{Trp}, that is, it was equivalent to "spot 1" in previous analyses (7). When it was eluted from the gel and digested with ribonuclease T₁, and the digest was separated by the method of Sanger *et al.* (25), its fingerprint was identical to that of tRNA^{Trp}. The fingerprints of the two other tRNAs in Fig. 3b were similar to each other and to that of "spot 6" (7). This tRNA appears to be tRNA^{Met} based on its fingerprint and minor nucleotide analysis of its oligonucleotides (26, 27). The 2-dimensional electrophoretic profile of the tRNA not bound by reverse transcriptase (Fig. 3c) was similar to that of whole tRNA; it still contained some unbound tRNA^{Trp} as identified by the fingerprint of the tRNA eluted from the region marked by an arrow in Fig. 3c. It should be noted that the two spots with low mobility in both dimensions in Fig. 3a were 5S RNA and are absent from Fig. 3b and c probably because they eluted between the two fractions pooled from the Sephadex columns (see Fig. 2).

Two-dimensional electrophoretic separation of the virion-associated tRNAs (Fig. 3d), the fraction bound by reverse transcriptase (Fig. 3e), and the unbound fraction (Fig. 3f) showed that the enzyme selected a portion of the virion-associated tRNAs. Again, tRNA^{Trp} was the major species bound by the enzyme (arrow in Fig. 3e) as determined by fingerprint analysis. Some of the minor spots of bound tRNA appeared to be tRNA^{Met}. Also, some tRNA^{Trp} was still present in the unbound tRNA (arrow in Fig. 3f).

Rate Zonal Centrifugation of Reverse Transcriptase-tRNA Complexes. To further characterize the complex between reverse transcriptase and tRNA^{Trp}, we measured the sedimentation coefficient of the complex, using glycerol gradients. The enzyme was detected in the gradients by its DNA polymerase activity. The free enzyme had a sedimentation coefficient of 7.2 S (Fig. 4A) by comparison to sedimentation rate of a marker *E. coli* DNA polymerase I that has a sedimentation coefficient of 5.8 S (22). Mixing of the reverse transcriptase with a molar excess of unlabeled purified tRNA^{Trp} (0.5 μ g) increased the sedimentation rate of the enzyme to 9 S (Fig. 4B). Addition of unfractionated chicken liver tRNA (60 μ g) similarly increased the sedimentation rate of the enzyme to 9 S (Fig. 4C). *E. coli* DNA polymerase I in the same gradients did not change its sedimentation rate upon addition of tRNA. To examine the effect of reverse transcriptase on the sedimentation rate of tRNA^{Trp}, we mixed [³²P]tRNA^{Trp} (approximately 0.01 μ g, 6800 cpm) with the enzyme and analyzed by centrifugation. Most of the enzymatic activity sedimented at the rate of free reverse

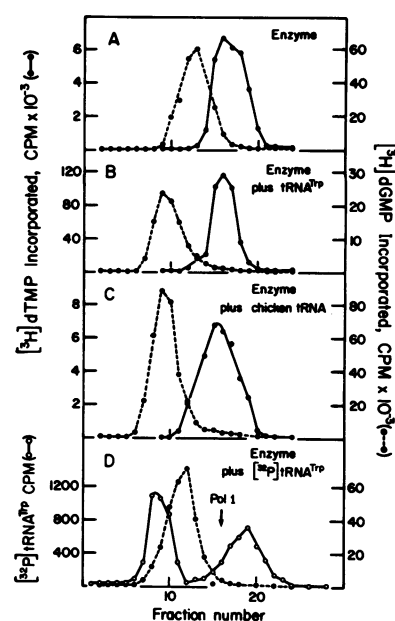


FIG. 4. Sedimentation through glycerol gradients of mixture of tRNA^{Trp} and reverse transcriptase. Reaction mixtures of 200 μ l with 37.5 units of reverse transcriptase were prepared as in gel filtration analysis. After 10 min incubation at 4°, *E. coli* DNA polymerase I (Pol I, 6 units, ref. 22) was added as a marker and the mixtures were layered onto 5 ml of 20–40% v/v glycerol gradients in 0.1 M potassium phosphate (pH 7.5), 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.1% Nonidet P-40, and 0.1 mM EDTA. After centrifugation for 26 hr at 46,000 rpm in an SW50.1 Spinco rotor, the gradient was fractionated into 0.17 ml fractions and assayed for reverse transcriptase, *E. coli* DNA polymerase I, and [³²P]tRNA. (A) Reverse transcriptase alone; (B) reverse transcriptase plus 0.5 μ g of cell tRNA^{Trp}; (C) reverse transcriptase plus 60 μ g of total chicken liver tRNA; (D) reverse transcriptase plus 5000 cpm cell [³²P]tRNA^{Trp}. ●-●, reverse transcriptase; ○-○, *E. coli* DNA polymerase I; ○-○, [³²P]tRNA.

transcriptase because very little tRNA^{Trp} was added. Fifty-two percent of the added [³²P]tRNA^{Trp} sedimented at 9 S, in a position corresponding to the unlabeled tRNA^{Trp}-enzyme complex formed by adding excess tRNA^{Trp}. Unbound [³²P]tRNA sedimented at 4 S. The ability of tRNA^{Trp} to increase the sedimentation rate of reverse transcriptase may explain the observation of Duesberg *et al.* (29) that reverse transcriptase isolated from virions has a faster sedimentation rate than the purified enzyme.

The ability of tRNA^{Trp} to increase the sedimentation rate of reverse transcriptase indicates that the tRNA binding activity of the enzyme preparation is not due to a contaminant. Furthermore, no tryptophanyl tRNA synthetase, the most likely contaminant that could be responsible for the binding (30), could be found in our enzyme preparations (unpublished data). Also, anti-reverse transcriptase IgG prepared from the serum of a rat immunized with pure AMV reverse transcriptase (18), but not nonspecific IgG, inhibited both DNA polymerase activity and the tRNA^{Trp} binding activity (data not shown).

DISCUSSION

The ability to selectively bind tRNA adds another activity to those already defined on the reverse transcriptase molecule. These include a ribonuclease H activity (31, 32) and a DNA polymerase able to copy both RNA and DNA (2). The DNA polymerase activity presumably requires at least three sites

(33): a template-binding site, a deoxyribonucleoside-triphosphate-binding site, and a site for recognition of the 3'-OH group of a growing polydeoxyribonucleotide chain. Because the sites involved in both ribonuclease H and DNA polymerase activity do not have the necessary specificity, the tRNA^{Trp}-binding site must be different from the others. Its function could be to position the 3'-OH at the -C-C-A OH end of the tRNA in the normal 3'-OH binding site, but more detailed work will be necessary to elucidate the details of its function in the initiation of DNA synthesis on 70S RNA.

From the present data and earlier reports it is evident that tRNA^{Trp} has two critical specificities which allow it to function as the primer of the DNA copies of 70S RNA. It has affinity for a site or sites on both 35S RNA (12, 13) and on reverse transcriptase. The virus must have evolved to utilize the tRNA in these two ways so as to provide a mechanism for initiation of DNA synthesis. While there is still no evidence that tRNA^{Trp} initiates reverse transcription *in vivo*, existence of a high affinity site for tRNA^{Trp} on the enzyme strongly argues that tRNA^{Trp} does initiate *in vivo* DNA synthesis. Our present evidence relates only to avian viruses; the binding properties of the reverse transcriptase from murine viruses remain to be studied.

Many tRNA molecules occur in virions of RNA tumor viruses as "free" tRNA, that is, not bound in the 70S RNA complex (10). The binding of the tRNA^{Trp} and tRNA^{Met} to reverse transcriptase can explain why these tRNAs are found in virions, but these two tRNA species represent only 30% of the virion-associated tRNA (Fig. 2D) and the reason for inclusion of the other tRNA species still requires explanation.

Purified tRNA^{Trp} from chick embryo fibroblasts is only partially bound to reverse transcriptase even when a large excess of enzyme is used. No structural difference between the bound and unbound fractions has as yet been detected. It could be that conformational isomers of tRNA^{Trp} explain the two fractions or that minor structural differences are present. The clear separation of the bound and unbound fractions after either Sephadex G-100 chromatography or glycerol gradient centrifugation suggests that the binding affinity is very high and that dissociation of the tRNA-enzyme complex during analysis is not responsible for the two fractions.

The interaction of tRNA^{Trp} and reverse transcriptase is reminiscent of the binding of tRNA to its cognate aminoacyl tRNA synthetase (34). Both binding reactions occur rapidly at low temperatures, can be analyzed by gel filtration, and are very selective (34, 35). Both reverse transcriptase and synthetase bind deacylated as well as aminoacylated tRNA (unpublished results).

It was pointed out previously that the initiation of DNA synthesis by a preformed RNA primer may occur in systems other than the RNA tumor viruses (3). One possible way to identify such primers would be to show binding of an RNA to a cellular DNA polymerase.

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